User Guide:
ImageCytox – Fiji/ImageJ macro for nuclei-based quantification of image-based 3D cytotoxicity assays

1. Overview

The macro performs nuclei-based segmentation and classification to quantify surviving tumor cells, dead/apoptotic nuclei, T cells (optional) and excluded objects, and generates both numerical output and visual quality-control overlays.

Key features:

- Automated nuclei segmentation using StarDist
- Classification of nuclei into:
  - viable tumor cells
  - apoptotic/dead tumor cells
  - T cells *(optional)*
- Batch processing of entire experiment folders
- Automatic generation of:
  - a summary CSV file
  - a consolidated image stack with color-coded overlays for quality control and documentation of segmentation and classification

2. Software Requirements

- Fiji/ImageJ (tested with Fiji ≥ 2.14)
- Installed plugins:
  - StarDist 2D
  - CSBDeep
  - Java 8 or newer (bundled with Fiji)

Note: StarDist installation issues can occur depending on Fiji and TensorFlow versions. We recommend using a Fiji installation where StarDist segmentation can be successfully run on a single test image before batch analysis.

3. Input Data Requirements

3.1 Folder structure

The macro expects the following directory layout:

```
TOP_FOLDER/
│
├── Medium/
│   ├── well-F03_pos-1.tif
│   ├── well-F03_pos-2.tif
│   └── ...
│
├── ET ratio 2-1/
```

```
│   ├── well-F05_pos-1.tif
│   └── ...
│
├── ET ratio 1-1/
│   └── ...
│
└── Results/      (created automatically by the macro)
```

Each subfolder corresponds to one experimental condition.

3.2 Image format

- Multichannel TIFF hyperstacks
- One file per field of view
- Channels must include:
  - Hoechst (nuclei; or other DNA/nuclei staining)
  - Propidium iodide (dead cells; or other marker to label dead cells)
  - Optional: T cell marker (e.g. GFP)
- The macro will ask the user to specify the channel indices at runtime.

4. Running the Macro

1. Open Fiji
2. Open the macro file:
   Plugins → Macros → Run...
3. Select the top-level folder containing all condition subfolders
4. In the dialog window:
   - Specify channel indices (1-based)
   - Define parameters and thresholds for nuclei classification
5. Start batch processing

5. Output

All results are written to:

```
TOP_FOLDER/Results/
```

5.1 Summary CSV

```
CytoxAssay_summary.csv
```

The summary CSV file contains one row per analyzed image and includes the following information:

- Condition (folder name)
- Position (image file name)
- Survivors (viable tumor nuclei)
- TotalROIs (total number of segmented nuclei)
- DeadNuclei (PI-positive nuclei)
- T-Cells (nuclei classified as T cells based on marker intensity and size)
- HoechstBright (condensed Hoechst-bright nuclei excluded from survivors)
- Fragments/Other (objects excluded based on size or morphology)

In addition, a SanityCheck section reports:

- NucleiMeanIntensity (reference mean intensity based on the intensity of healthy nuclei)
- NucleiIntThreshold (Hoechst-bright exclusion threshold, reference intensity x user-defined factor)
- PI_BG_Intensity (background intensity in the PI channel, used to calculate the PI threshold based on the background intensity and a user-defined factor)
- MedianNucleiSize

5.2 Quality Control (QC) image stack

CytoxAssay_Results_stack.tif

The QC image stack is a multi-slice RGB TIFF file containing one slice per analyzed image. Nuclear outlines are color-coded as follows:

- White (thick outline): surviving tumor nuclei
- Magenta: dead/apoptotic nuclei
- Orange: T cells
- Cyan: Hoechst-bright condensed nuclei
- Gray (thin outline): excluded objects (too small or too large nuclei-ROIs)

The QC stack enables rapid visual inspection of segmentation quality and classification accuracy across the entire experiment.

6. Interpretation & Quality Control

Users are strongly encouraged to test the macro and adjust the parameters by visually inspecting the QC stack on a small test dataset (1 folder containing controls without T cells, 1 folder containing images from a high ET ratio with T cells and apoptotic cells) before application to a full dataset.

Following the automated processing of the entire dataset, the QC stack allows to exclude images with poor segmentation, focus issues or staining artifacts from downstream analysis.

Important note on data interpretation:

The counts reported for excluded categories (e.g. DeadNuclei, T-Cells, HoechstBright, Fragments/Other) are provided for quality control and transparency only and should not be used to draw quantitative conclusions about absolute numbers of dead cells or T cells.

Several biological and technical factors limit the reliable quantification of these populations:

- PI-positive nuclei represent only cells that are dead at the imaging endpoint. Tumor cells that died earlier during the assay and subsequently disintegrated or detached are not captured and will be missed.
- Dead tumor cells frequently fragment, making it difficult or impossible to assign fragments unambiguously to a single original tumor cell.
- T cell detection is incomplete, particularly in 3D assays. T cells that are out of focus or weakly fluorescent can fall below intensity thresholds and are therefore not reliably detected.

For these reasons, ImageCytox is designed to use the number of surviving tumor nuclei (“Survivors”) as the primary quantitative readout. This metric is robust, reproducible and allows reliable comparison across experimental conditions, even when absolute counts of dead cells or T cells are incomplete.

7. Example datasets

This repository includes two example datasets that illustrate typical and challenging analysis scenarios. Both datasets were acquired using B16F10-OVA tumor cells and OT-I CD8 T cells.

Example dataset 1: ET ratios

This dataset contains three experimental conditions:

- Medium (no T cells)
- ET ratio 1:1
- ET ratio 2:1

Images contain three channels:

1. Nuclei (Hoechst)
2. Dead-cell marker (Propidium iodide)
3. Brightfield (ignored by the macro)

No T cell marker channel is present in this dataset.

The default settings of the ImageCytox macro are optimized for this dataset and can be used without modification.

Example dataset 2: Clustered cells

This dataset contains a single experimental condition (ET ratio 1:1) and represents more challenging imaging conditions. Tumor cells form dense clusters, which complicates nuclei segmentation and classification.

Images contain four channels:

1. Nuclei (Hoechst)
2. Dead-cell marker (Propidium iodide)
3. T cell marker (GFP-OT-I CD8 T cells)
4. Brightfield (ignored by the macro)

Imaging conditions differ from the ET ratios dataset, including pixel size and apparent nuclear size. Therefore, macro parameters must be adjusted as follows:

- Select the T cell marker channel (channel 3)
- Set the maximum T cell nucleus size to 80 pixels
- Keep the T cell intensity factor at the default value (1.2)
- Set tumor nucleus size thresholds to:
    - Minimum size: 40 pixels
    - Maximum size: 300 pixels

All other parameters can be left at their default values.

8. Citation

If you use ImageCytox, please cite the corresponding Zenodo record (DOI provided upon release).

A detailed description of the method is provided in the accompanying book chapter:

Ohmayer A., Giampetraglia M., Weigelin B. Real-time imaging of T cell-mediated cytotoxicity in a 3D collagen matrix assay. [Book / Publisher / Year].

9. Contact

For questions or issues, please contact:

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